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Mitochondrial compromise in 3 year old patas monkeys exposed *in utero* to human-equivalent antiretroviral therapies

Yongmin Liu¹, Eunwoo Shim Park¹, Alexander T. Gibbons¹, Eric D. Shide¹, Rao L. Divi², Ruth A. Woodward³, and Miriam C. Poirier¹

¹Carcinogen-DNA Interactions Section, Laboratory of Cancer Biology and Genetics, CCR, National Cancer Institute, NIH, Bethesda, MD

²Methods and Technologies Branch, DCPC, National Cancer Institute, NIH, Bethesda, MD

³NIH Animal Center, Shared Animal Facility, Dickerson, MD

Abstract

Antiretroviral (ARV) drug therapy, given during pregnancy for prevention of mother-to-child transmission of human immunodeficiency virus 1 (HIV-1), induces fetal mitochondrial dysfunction in some children. However the persistence/reversibility of that dysfunction is unclear. Here we have followed *Erythrocebus patas* (patas) monkey offspring for up to 3 years of age (similar in development to a 15 year old human) after exposure of the dams to human-equivalent *in utero* ARV exposure protocols. Pregnant patas dams (3–5/exposure group) were given ARV drug combinations that included zidovudine (AZT) / lamivudine (3TC) / abacavir (ABC), or AZT / 3TC / nevirapine (NVP), for the last 10 weeks (50%) of gestation. Infants kept for 1 and 3 years also received drug for the first 6 weeks of life. In offspring at birth, 1 year and 3 years of age mitochondrial morphology, examined by electron microscopy (EM), was compromised compared to the unexposed controls. Mitochondrial DNA (mtDNA), measured by Hybrid Capture Chemiluminescence Assay (HCCA) was depleted in hearts of patas exposed to AZT/3TC/NVP at all ages ($p < 0.05$), but not in those exposed to AZT/3TC/ABC at any age. Compared to unexposed controls, mitochondrial reserve capacity oxygen consumption rate (OCR by Seahorse) in cultured bone marrow mesenchymal fibroblasts from 3 year old patas offspring was ~50% reduced in AZT/3TC/ABC-exposed patas ($p < 0.01$), but not in AZT/3TC/NVP-exposed patas. Overall the data show that three year old patas sustain persistent mitochondrial dysfunction as a result of perinatal ARV drug exposure.

Keywords

zidovudine; lamivudine; abacavir; nevirapine; mitochondrial DNA; Seahorse analyzer

Corresponding author contact information: Miriam C. Poirier, Ph.D., Head Carcinogen-DNA Interactions Section, National Cancer Institute, NIH, Bldg. 37, Rm 4032, 37 Convent Drive, MSC-4255, Bethesda, MD, 20892-4255, Phone: 301-402-1835, FAX: 301-402-0153, poirierm@exchange.nih.gov.

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INTRODUCTION

Antiretroviral (ARV) drug combination therapy is highly successful, not only in controlling Human Immunodeficiency Virus-1 (HIV-1) infection, but also in preventing HIV-1 mother-to-child transmission during pregnancy. Because the transmission rate has been reduced to <1% (Siegfried *et al.*, 2011), UNAIDS and partners have set new goals, calling for virtual elimination of HIV-1 MTCT during the next few years (Heidari *et al.*, 2014; Mahy *et al.*, 2010). As we move to approach that goal we continue to have concerns regarding the toxicity of *in utero* antiretroviral drug exposures, especially in the HIV-1-exposed uninfected (HEU) children born to HIV-1-infected mothers (Andre-Schmutz *et al.*, 2013; Brogly *et al.*, 2007; Foster & Lyall, 2008; Poirier *et al.*, 2015). Both genotoxicity (Olivero *et al.*, 2013; Poirier *et al.*, 2004) and mitochondrial toxicity (Divi *et al.*, 2010; Divi *et al.*, 2005; Torres *et al.*, 2010) have been observed in experimental models upon transplacental exposure to various ARV drug combinations, and the frequently-used nucleoside reverse transcriptase inhibitor (NRTI) zidovudine (AZT) is a transplacental carcinogen in mice (Olivero *et al.*, 1997; Walker *et al.*, 2007). In HEU children born to mothers receiving ARV therapy during pregnancy, both genotoxicity, in the form of aneuploidy (Andre-Schmutz *et al.*, 2013), and mitochondrial dysfunction, in the form of cardiac abnormalities and neuromuscular dysfunction (Brogly *et al.*, 2007; Lipshultz *et al.*, 2013; Sibiude *et al.*, 2015) have been documented. Of particular concern in the children is the persistence of ARV-induced drug effects, and the primate study presented here was designed to investigate the persistence/reversibility of these effects.

The observed cardiovascular and neuromuscular sequelae, which may be due to the HIV-1 infection and/or the ARV therapy, are largely considered the result of mitochondrial events. In particular, NRTI exposures damage mitochondria by inhibiting DNA polymerase γ , the mitochondrial DNA polymerase, and also by becoming incorporated into mitochondrial DNA (mtDNA). Both events result in arrest of mtDNA replication (Lewis & Dalakas, 1995), the induction of mutagenesis in mitochondrial DNA, and loss of mitochondrial function. Similar degrees of mitochondrial damage have been observed in umbilical cords from both human and *Erythrocebus patas* (patas) monkey pregnancies, where oral NRTI dosing in the patas was designed to model that in the human (Divi *et al.*, 2007a). Because the patas monkeys are not susceptible to immunodeficiency virus infection, the mitochondrial compromise is due only to the ARV drug exposures.

In this study, we exposed pregnant patas dams to human-equivalent ARV drug combination protocols for the last half (10 weeks) of gestation to model use in human pregnancy. Offspring were examined at birth, 1 and 3 years of age, with patas development at 3 years considered equivalent to that of a 15 year old human adolescent. We chose the frequently-used combinations: AZT/lamivudine (3TC)/abacavir (ABC), where all three drugs are NRTIs; and, AZT/3TC/nevirapine (NVP), where NVP is a non-NRTI known to bind covalently to DNA (Antunes *et al.*, 2013). Having observed in previous studies that NRTI two-drug combinations induced mitochondrial compromise in patas infant heart (Divi *et al.*, 2005), skeletal muscle (Divi *et al.*, 2007b), brain and liver (Divi *et al.*, 2010), which was not reversed by one year of age, here we followed the offspring up to 3 years of age.

MATERIALS AND METHODS

Patas maintenance, drug sources, and exposure protocols

Maintenance of the patas has been previously described (Divi *et al.*, 2005; Divi *et al.*, 2007b). Monkeys were housed and treated under conditions approved by the American Association for the Assessment and Accreditation of Laboratory Animal Care International, using protocols reviewed by the Institutional Animal Care and Use Committee of Bioqual Inc., or the National Cancer Institute Animal Care and Use Committee. Euthanasia was performed in accordance with the 2013 American Veterinary Medical Association Guidelines. Monkeys were housed in groups of 1 male and 3 females, and pregnancy was assessed by ultrasonography. Pregnant dams (3–4/group) were given NRTIs during the final 50% (10 weeks) of gestation. Exposed and unexposed patas offspring were either taken near term by cesarean section (Divi *et al.*, 2005) or born naturally and permitted to grow to 1 or 3 years of age.

AZT was obtained from Sigma Chemical (St. Louis, MO), and the pediatric liquid clinical formulation of 3TC was from Glaxo-Wellcome (Raleigh, NC). ABC and NVP clinical formulations came from the National Institutes of Health Veterinary Pharmacy (Bethesda, MD). Each drug was dissolved in syrup, which was placed inside of a banana or mixed into pudding, and given as a treat, under supervision, to the pregnant patas. AZT and ABC were given for the last 10 weeks of gestation, in two daily 20-mg doses, for a total of 40 mg/day 5 days/week. 3TC and NVP were given for the last 4 weeks of gestation in two daily 12-mg doses, for a total of 24 mg/day 5 days/week. Unexposed monkeys received bananas containing syrup twice daily for 10 weeks. To model human clinical ARV use, in which infants receive NRTI therapy for 6 weeks after birth, patas neonates to be observed for 1 and 3 years received either the drug combination or placebo in liquid formulation twice daily by oral syringe, for the first 6 weeks of life (Divi *et al.*, 2005).

The tissues used for these analyses were taken at autopsy from the heart left ventricle and the brain frontal cortex of each animal. Values for the actual numbers of patas used for each type of experimental end point varied between 2 and 5 per exposure group, and are presented in the Figure Legends.

Mitochondrial morphology determined by Electron Microscopy (EM)

Heart and brain tissues from ARV drug-exposed patas were taken at birth, 1 year or 3 year of age, and the same tissues from unexposed patas were taken at birth and 1 year. In this study we are lacking heart and brain from 3 year old unexposed patas because of the ethical complications of euthanizing otherwise-healthy animals that were necessary for breeding. We realize the bias introduced by comparing 1 year old controls with 3 year old exposed patas, but it was our opinion that mitochondria in young hearts and brains are not likely to undergo significant compromise between the ages of 1 and 3 years. Tissues were processed as previously described (Divi *et al.*, 2010; Divi *et al.*, 2005). Briefly, tissues were cut into 1- to 2-mm slices and fixed, for 24 hr at 4°C, in 4% paraformaldehyde containing 2% glutaraldehyde in 100mM phosphate buffer, pH 7.4. The samples were kept at 4°C and further processed to obtain EM photomicrographs (Leidos Laboratory, Frederick, MD).

Images were captured randomly in a “Z” pattern, and 10 photomicrographs ($\times 30,000$ magnification)/organ/animal were obtained. The photos (30–40/treatment group) were coded, and then scored by three independent investigators. The degree of mitochondrial pathology was evaluated using a scoring paradigm of 0 to 5 (presented in the legend to Figure 1), where 0 represents undamaged mitochondria and 5 represents the highest level of damage (Divi *et al.*, 2005).

Mitochondrial DNA quantification in patas heart by Hybrid Capture Chemi-luminescence Assay (HCCA)

The HCCA was performed on 3–5 patas hearts per treatment group, with treatment groups including control, AZT/3TC/NVP and AZT/3TC/ABC taken at birth, 1 year and 3 years of age (9 groups in total). DNA was extracted from patas hearts using the Qiagen DNeasy Blood and Tissue Kit, according to the manufacturer’s protocol (Qiagen, Valencia, CA). Briefly, tissues were equilibrated to room temperature, sliced into small pieces, washed with 100mM PBS without calcium and magnesium (pH 7.4), homogenized in three volumes of PBS, lysed with Qiagen AL buffer, and digested with proteinase K and RNase A. The DNA was separated using spin columns provided with the kit, and was subsequently extracted into 10mM Tris, pH 7.4, containing 1mM EDTA and stored at -80°C until analysis. The quality and quantity of DNA were determined using a NanoDrop Spectrophotometer.

The protocol for HCCA is described in detail elsewhere (Divi *et al.*, 2005) and so will be outlined only briefly here. Sample DNA containing both nuclear and mtDNA was mixed with a 500-bp amplicon of the human cystic fibrosis (CFTR-Human) gene prepared by PCR amplification using specific primers (Ensembl gene ENSG0000001626; forward: 5′-AAGCTTCAGATCACTGTGGAAGAGG; reverse: 5′-GACTTGCACTTGCTTGAGTTCCG) and the human genomic DNA as template. The CFTR-human DNA was used as a nonspecific carrier matrix so that all samples would have a total of 1 μg DNA. Diluted samples were mixed with 0.1 μM patas monkey complex I (NADH dehydrogenase) ND4/ND5 (5′-amino-TTCTCATAATCGCCCACGGA) (GenBank ID: [D85291](#)) oligonucleotide (Invitrogen, Carlsbad, CA) in 10 μl of Expand High Fidelity PCR buffer without MgCl_2 (2 X concentration, Roche Diagnostics) and denatured together at 95°C for 5 min followed by hybridization at 60°C for 30 min. The hybrid was labeled with 1.0 μl of ULS Platinum Bright (Applied Biosystems, Foster City, CA) at 55°C for 1 h. The Platinum Bright-hybridized DNA was separated from unhydrolyzed oligonucleotide and unreacted Platinum Bright using YM-10 or YM-100 microcon spin columns (Millipore, Billerica, MA) and washing with binding buffer (10mM phosphate buffer, pH 7.4, with 1mM EDTA).

The purified hybrid was diluted to 0.5 ml in binding buffer, and an aliquot (100 μl) in four replicates was transferred to a DNA-Bind (amide-binding N-oxysuccinamide surface) 96-well plate (Corning, Acton, MA) and incubated at 37°C for 1 h to capture the hybrid. The plates were washed once with wash buffer (2 $\times$ saline sodium citrate, 0.1% SDS) and three times with PBST (PBS with 0.05% Tween 20). They were blocked with 0.25% I-Block (Casein; Applied Biosystems) in PBS for 1 h at 37°C and again washed three times with PBST. Alkaline phosphatase (AP; Applied Biosystems), diluted 1:2000 in I-block buffer,

was added to all the wells and incubated for 1 h at room temperature before wells were washed with PBST and rinsed with 10mM Tris buffer (pH 8.0). Following incubation with an AP substrate (CDP-Star with Emerald II; Applied Biosystems) for 30 min at room temperature, luminescence was measured using a TR 717 Luminometer (Applied Biosystems). The values for mtDNA quantity are expressed as luminescence units (LUs) per nanogram of total patas DNA, with unexposed samples compared to exposed samples.

The HCCA was performed 3 times on each patas heart DNA sample and the results averaged to obtain a value for each animal. The patas in each treatment group were then averaged and the results expressed as mean $\pm$ SEM.

Patas bone marrow culture and measurement of mitochondrial oxygen consumption rate (OCR) by Seahorse XF24 Analyzer

Using sterile technique we collected bone marrow from the femurs of 3–4 year old patas, and cultured the cells in T75 flasks (BD Falcon, Bedford, MA) with Roswell Park Memorial Institute 1640 medium and 10% inactivated fetal bovine serum (American Type Culture Collection, Manassas, VA). Harvested cells were incubated undisturbed for 72 hours, at which time the medium was changed, and colonies of adherent mesenchyme-derived cells were allowed to expand. Subsequently cells were subcultured, and equal numbers of bone marrow cells were seeded in quadruplicate wells of XF24 V7 cell culture plates (Seahorse Bioscience, North Billerica, MA). After 12 h, the medium was replaced with XF Assay Medium (Seahorse Bioscience), supplied with 0.9% glucose, 1mM sodium pyruvate, and 2mM glutamine. After 1 h of incubation in a non-CO₂ incubator, OCR was measured using the Seahorse Bioscience XF24 Extracellular Flux Analyzer (Seahorse Bioscience) where cells were subjected to the following sequence of conditions (Steps): (1) basal OCR was measured with no additives; (2) 1 μ M oligomycin (OLIGO), which reversibly inhibits ATP Synthase and oxidative phosphorylation (OXPHOS), was added to show glycolysis alone; (3) 0.3 μ M carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP), a mitochondrial uncoupler, was added to induce maximal respiration; (4) 0.1 μ M rotenone (ROT), a Complex I inhibitor and mitochondrial poison, was added to end the reaction. Three sequential measurements were taken after each of the above reagents was added. For each Seahorse experiment maximal OCR (determined in Step 3) was measured in quadruplicate wells/ experimental group, and the results were plotted by Seahorse software. The Seahorse experiment was repeated four times on separate occasions using different cell cultures. The study included 2 unexposed patas, 3 patas exposed to AZT/3TC/NVP, and 2 patas exposed to AZT/3TC/ABC.

Statistical analysis

The actual numbers of patas used for each type of experiment are shown in the Figure Legends, but typically 3–5 patas were used per exposure group. For a number of reasons our statistical advisors in previous patas studies have indicated that ANOVA is an appropriate approach. The EM, mtDNA and Seahorse maximal OCR data are all presented as mean $\pm$ standard error of the mean (SEM). Statistical comparisons employed one-way ANOVA, where significance is presented as * for $p < 0.05$, or ** for $p < 0.01$.

RESULTS

Mitochondrial morphology

Mitochondrial morphology, analyzed by EM, was examined in heart and brain of patas offspring taken at birth, 1 year and 3 year of age. There were 3–4 patas / treatment group, and representative photomicrographs are shown. Patas at birth are shown in Figure 1 (heart in A, and brain in C), and patas at 1 and 3 years of age are shown in Figure 1 (heart in C, and brain in D). Figure 1A shows normal unexposed heart (Control), and damage (black arrows) in heart mitochondria from patas exposed perinatally to the combinations AZT/3TC/NVP and AZT/3TC/ABC. Figure 1C shows unexposed heart, and damaged heart mitochondria (black arrows) from 1 and 3 year old patas. Similarly, EM photomicrographs from brains can be seen in Figure 1, where B shows patas brain at birth, and (D) shows brains from 1 and 3 year old patas, and damage from the triple ARV drug combinations are indicated by black arrows. Morphological manifestations of mitochondrial compromise (indicated by black arrows), seen at all ages in the AZT/3TC/ABC and AZT/3TC/NVP groups, included mitochondrial swelling and disorganization, membrane breaks, loss of cristae with matrical lucency, and increased aberrations of the surrounding structures, including heart muscle sarcomeres and brain endoplasmic reticulum.

In order to obtain semi-quantitative values indicative of the level of mitochondrial damage, EM photomicrographs (30–40/group) were coded and scored using a paradigm (see the Legend to Figure 2) where undamaged mitochondria scored 0, and mitochondria with the highest level of damage scored 5. Three investigators evaluated the coded photos, and the results are shown in Figure 2(A, B, C, and D). At birth, mitochondrial compromise in the patas hearts (Figure 2A) and brains (Figure 2B), of the AZT/3TC/ABC and AZT/3TC/NVP exposure groups, did not reach statistical significance, compared to unexposed patas, except for brains of the AZT/3TC/ABC-exposed patas ($p < 0.05$). For analysis of the 1 and 3 year age groups, the 1 year unexposed patas were used as the control. For both heart (Figure 2C) and brain (Figure 2D) there was significantly-increased ($p < 0.05$) mitochondrial morphological damage in most of the ARV-drug exposed 1 and 3 year old patas, compared to the unexposed groups. The one exception is the 1 year old AZT/3TC/NVP-exposed patas brains (Figure 2D) where the damage did not increase significantly. Therefore, semi-quantitative scoring showed that heart and brain mitochondrial morphology did not recover during the first 3 years of life, even though the last drug exposure was at 6 weeks of age.

Mitochondrial DNA quantitation in patas heart at birth, 1 and 3 years of age

Another indicator of mitochondrial integrity, quantity of mtDNA, was analyzed by HCCA. Because incorporation of NRTIs into mtDNA truncates DNA replication, we expected to find mtDNA depletion. Using patas heart (Figure 3), we found that whereas the AZT/3TC/ABC treatment did not alter patas mtDNA levels at any age, the AZT/3TC/NVP treatment significantly reduced mtDNA levels, compared to untreated controls, at birth, 1 and 3 years of age ($p < 0.05$ at each time point). The data suggest that different drug combinations may have a variety of effects, despite the similar modes of action.

Mitochondrial function in 3–4 year old patas

To determine more directly the effects of NRTI exposure on patas mitochondrial respiration, we employed the Seahorse Analyzer to measure OCR in patas mesenchymal cells grown from bone marrow aspirate. Cells were obtained from patas offspring exposed to no drug, AZT/3TC/ABC or AZT/3TC/NVP *in utero* and for 6 weeks after birth, and cultured cells were subjected to the typical Seahorse sequence of treatments, which included OLIGO, FCCP and ROT. A representative Seahorse profile is shown in Figure 4, where the maximal OCR levels, induced by uncoupling with FCCP, are reduced in the patas exposed to AZT/3TC/ABC. Here, FCCP caused uncoupling and allowed measurement of the maximal OCR. The summary data are presented in Table 1, and show that cells from patas exposed to AZT/3TC/NVP had apparent reductions in basal and glycolytic respiratory rates, though no reduction in reserve capacity was found. Cells from patas exposed to the triple NRTI combination AZT/3TC/ABC had substantial reductions in basal rate and glycolysis, as well as striking reductions in maximal respiratory capacity ($p<0.01$) and reserve capacity (Table 1). The fact that 3 year old patas, exposed to AZT/3TC/ABC for the last half of gestation and the first 6 weeks of life, sustained a ~50% loss of mitochondrial respiratory capacity in bone marrow cells (Supplementary Figure 1), speaks to the long-term persistence of ARV drug induced mitochondrial compromise.

DISCUSSION

Using a breeding colony of patas monkeys, in which human-equivalent ARV therapy was administered to the dam for the last half of gestation and to the infant during the first 6 weeks of life, we modeled the fetal effects of NRTI-based ARV therapy received by HIV-1-infected pregnant women in the clinic. Because these drugs become incorporated into both nuclear and mitochondrial DNA causing genotoxicity (Olivero *et al.*, 2013; Poirier *et al.*, 2015) there is concern for both mitochondrial compromise and cancer risk as long-term sequelae. In this study we report mitochondrial compromise occurring in hearts, brains and bone marrow mesenchymal cells of the drug-exposed infants, and persistence of mitochondrial structural and functional abnormalities in heart and brain for up to 3 years of age. Patas infants, at 3 years of age, are sexually mature and developmentally equivalent to a 15 year old human adolescent. Therefore, the long-term persistence of morphological and biochemical mitochondrial compromise in the patas suggests that children who are born HIV-1-exposed but uninfected (HEU) may sustain sequelae, derived from perinatal ARV drug exposures, that persist into the adolescent years.

In patas offspring exposed to AZT/3TC/NVP and AZT/3TC/ABC, combinations frequently used in human pregnancy, EM photomicrographs revealed persistent mitochondrial morphological damage in the heart and brain at 1 and 3 years of age, compared to 1 year old unexposed controls. A disadvantage of this study design is the lack of 3 year old patas controls, however, we were reluctant to euthanize healthy 3 year old animals that were needed for breeding, and we therefore chose to use 1 year old patas controls.

In the ARV drug exposed patas, we found that additional end points indicative of mitochondrial compromise were positive at birth, 1 year and 3 years of age. For example, the quantity of heart mitochondrial DNA was significantly reduced at all three time points in

the patas exposed to AZT/3TC/NVP, but not in those unexposed, or exposed to AZT/3TC/ABC. In addition, when mitochondrial function was examined in cultured bone marrow mesenchymal cells from 3 year old patas, the offspring exposed *in utero* to AZT/3TC/ABC had significantly impaired (~50% loss) reserve respiratory capacity, while those exposed to AZT/3TC/NVP had OCR levels similar to the unexposed controls. The data suggest that the manifestation and degree of mitochondrial toxicity may vary depending on the tissue and the drug combination under evaluation. Furthermore, NRTI-DNA incorporation is not the only potentially-relevant mechanism. Both oxidative damage (Glover *et al.*, 2014) and endothelial cytotoxicity mitigated by mitophagy (Xue *et al.*, 2013) contribute to NRTI-based mitochondrial toxicity. Nevertheless, both of these triple ARV drug combinations imparted some degree of mitochondrial compromise that was persistent up to 3 years of age.

The patas transplacental ARV drug exposures used here were designed to model human clinical protocols. The daily doses given are essentially equivalent, on a gm/kg body weight, to those received by HIV-1-infected pregnant women. These studies differ from our previous NRTI experiments, using the patas model, in that here we employed triple drug combinations and followed the offspring for up to 3 years of age, whereas previously NRTI 2-drug combinations were used and offspring were followed for 1 year (Divi *et al.*, 2010; Divi *et al.*, 2005; Divi *et al.*, 2007b). In designing this study, we hypothesized that the mitochondrial compromise seen at birth and 1 year would be reversible by 3 years of age, but the contrary seems to be true.

As with all experimental models, there is not perfect correspondence with the human situation in these studies. One weakness is the post-birth drug dosing time. We chose 6 weeks because that is the treatment time during which ARV drugs are given to children. The comparison of developmental differences in the patas and human infants at these young ages is difficult to approximate, and a 6 week patas may be more similar to a 3–4 month human infant. However, with no solid data available we gave the drugs for 6 weeks, considering that growth to 1 and 3 years of age would be sufficient time for reversibility of toxicity to occur. An additional weakness is the lack of unexposed 3 year old patas in the EM studies, as we were reluctant to euthanize healthy animals that could be used for breeding. Studies in humans indicate that aging related genotoxicity (Michikawa *et al.*, 1999) and mitochondrial compromise (Short *et al.*, 2005) are not significant until well into middle-age, and even very old rats do not sustain significant loss in mitochondrial integrity provided they maintain sufficient physical activity (Hepple, 2016). Therefore, we assumed that a 3 year old patas would have similar mitochondrial morphology to a 1 year old patas, and the 1 year old patas controls were used for both.

In previous studies using the patas model we had no means of evaluating mitochondrial function, however here we were able to assess oxidative phosphorylation capacity as OCR in bone marrow cells using the Seahorse Analyzer. The tissue chosen was based on a requirement of the Seahorse to use cultured cells, and the fact that patas bone marrow cells grow well in culture. The data obtained, using bone marrow taken from 3 year old patas offspring, showed reduced maximal and reserve respiratory capacity in uncoupled mitochondria from patas exposed to AZT/3TC/ABC, but not AZT/3TC/NVP. Again,

different combinations may produce different effects, but the most important point is that some of the sequelae of ARV drug exposure are persistent through primate adolescence.

The NRTIs become incorporated into both nuclear and mitochondrial DNA and truncate DNA replication, causing genotoxicity as well as mitochondrial toxicity. Previously, we evaluated genotoxic end points in cultured bone marrow cells from these same animals (Olivero *et al.*, 2013). Cells cultured from patas at birth, 1 and 3 years of age showed significantly-increased levels of centrosomal amplification, micronuclei, and aneuploidy when exposed to AZT/3TC/ABC and AZT/3TC/NVP, compared to the unexposed controls. A study of chromosomal aberrations in karyotypes of cord blood lymphocytes from children born to HIV-1-infected mothers (Andre-Schmutz *et al.*, 2013), showed a significantly-increased aneuploidy rate in children of mothers receiving AZT therapy, suggesting that the genotoxic events observed in the patas may have a human counterpart. The patas studies provide an important link to elucidating the contribution of drug and HIV-1 infection in humans. The patas are not susceptible to immunodeficiency virus infection, but as the patas bone marrow cells sustain both genotoxicity and mitochondrial toxicity when exposed perinatally to ARV drugs, it would appear that the drugs alone are capable of inducing mitochondrial compromise.

There is ongoing clinical monitoring of cohorts of HEU children born to HIV-1-infected mothers, and these studies have revealed minor degrees of biological compromise that may be due to the NRTI exposures. Cardiac clinical changes have been reported both in the United States (Lipshultz *et al.*, 2011; Lipshultz *et al.*, 2015) and in France (Sibiude *et al.*, 2015), and one study of cardiac biomarkers showed that among 338 HEU children 51% had at least one elevated cardiac biomarker (Wilkinson *et al.*, 2013). Additionally, there are reports of blood lactate elevation (Crain *et al.*, 2011), alterations in neurodevelopment and cognitive outcomes (Kerr *et al.*, 2014), and suspected changes in immune function (Afran *et al.*, 2014; Reikie *et al.*, 2014). Most of these studies involve young children, and most have not yet followed subjects into their young adult years. However, the presence of sensitive biomarkers in clinically asymptomatic children should not be overlooked, as the patas studies have shown evidence of progressive toxicity (Poirier *et al.*, 2015).

The persistence of NRTI-induced mitochondrial compromise in children exposed to ARV drugs *in utero* suggests that mitigation and/or prevention of mitochondrial impairment may be desirable clinical goals. Recently we demonstrated by Seahorse that AZT/ddI exposure reduced OCR in rat H9c2 cardiomyocytes (Liu *et al.*, 2012), and concomitant addition of Tempol, a stable free radical, increased OCR in the AZT/ddI-treated cells, protecting them from mitochondrial damage (Jiang *et al.*, 2014). Until there is a proven approach for mitigation of ARV drug induced mitochondrial toxicity, long-term monitoring of ARV drug exposed children would appear to be important for identification of individuals with potential adverse effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABC	abacavir
AP	alkaline phosphatase
ARV	antiretroviral
AZT	zidovudine
CFTR	human cystic fibrosis gene
3TC	lamivudine
EM	electron microscopy
FCCP	carbonyl cyanide <i>p</i> -trifluoromethoxy-phenylhydroazone
HCCA	hybrid capture chemiluminescence assay
HEU	HIV-1-exposed uninfected
HIV-1	Human Immunodeficiency Virus-1
LU	luminescence unit
mtDNA	mitochondrial DNA
NRTI	nucleoside reverse transcriptase inhibitor
NVP	nevirapine
OCR	oxygen consumption rate
OLIGO	oligomycin
OXPHOS	oxidative phosphorylation
PBS	phosphate buffered saline
ROT	rotenone

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Fig. 1.

Representative EM photomicrographs from hearts (A) and brains (B) of patas infants taken at birth, and hearts (C) and brains (D) of patas offspring taken at 1 and 3 years of age. Pregnant patas dams were exposed to no drug, AZT/3TC/NVP or AZT/3TC/ABC for the last 10 weeks (50%) of gestation, and infants to be kept for 1 or 3 years were given the same drugs for the first 6 weeks of life. The black arrows indicate damaged mitochondrial membranes and loss of cristae.

Fig. 2.

Mitochondrial EM damage scoring results from patas hearts (A) and brains (B) taken at birth, and patas hearts (C) and brains (D) taken at 1 and 3 years of age. Mitochondrial integrity was scored using a paradigm where 0 = normal mitochondria and 5 = the most highly-damaged mitochondria. Each data point represents mean $\pm$ SE for 3–4 patas/group, where 10 photomicrographs/monkey ($n=30$ –40 photos/experimental group) were coded and scored by 3 investigators. The scoring paradigm was as follows: (0) virtually-all mitochondria had the typical oblong shape, with compact, intensely dark, intact cristae, while an infrequent mitochondrion showed minimal swelling that was considered to be within normal limits; (+1) most mitochondria had the typical oblong shape, and although a few were rounded with discontinuous membranes no loss of cristae and matrix material were evident; (+2) the number of mitochondria per cell was increased compared with samples graded (0) and (+1), and many mitochondria were swollen with membrane disruptions but minimal loss of cristae, while most of the remaining mitochondria looked essentially normal; (+3) the number of mitochondria per cell indicated substantial mitochondrial proliferation, but most mitochondria were swollen with multiple membrane breaks and had partial loss of cristae and matrix material; (+4) mitochondrial proliferation was increased and virtually all mitochondria were swollen with fragmented membranes and effacement of central architecture, while clusters of highly-damaged, similar-appearing, mitochondria could be identified; (+5) in some areas the mitochondrial membrane was completely

dissolved and the cristae were fragmented and disorganized, in other areas giant swollen mitochondria containing no cristae or central architecture were apparent, and all other areas looked like those in (+4). Assuming that 1 and 3 year unexposed patas would have similar morphology, 1 year unexposed patas were used as controls for both the 1 and 3 year experiments. Statistical analysis was by one way ANOVA (* $p < 0.05$).

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Fig. 3.

Heart mtDNA levels were determined by HCCA, in 3–5 patas offspring per treatment group, taken at birth, 1 year, and 3 years of age after *in utero* and/or perinatal exposure to no drug, AZT/3TC/ABC or AZT/3TC/NVP. The HCCA was repeated 3–4 times to obtain a mean for each monkey. In the Figure, the bars represent mean $\pm$ SE for the group of monkeys (n=3–5) in each treatment group. One-way ANOVA was used to perform the analysis (* $p < 0.05$).

Fig. 4.

Representative Seahorse profile for Oxygen Consumption Rate (OCR) in patas bone marrow fibroblasts taken from 3 year old patas offspring exposed perinatally to no drug (◆), AZT/3TC/NVP (■), or AZT/3TC/ABC (▲), and grown in culture. Confluent cells in Seahorse plates (3–4 wells/sample) were incubated sequentially with: 1) no additives, to observe basal levels (1–20 minutes); 2) OLIGO, to visualize glycolysis (21–44 minutes); 3) FCCP, to observe maximal or uncoupled capacity (45–79 minutes); and 4) ROT, to end the reaction (80–96 minutes). OCR values for the unexposed controls, and the AZT/3TC/NVP-exposed group, were significantly ($p < 0.01$) higher than for cells from the AZT/3TC/ABC group.

Table 1

Seahorse Oxygen Consumption Rate (OCR, pMol/min, mean $\pm$ SE^a) and Related Parameters for 3 Year Old Patas Perinatally Exposed to No Drug (Control), AZT/3TC/NVP or AZT/3TC/ABC.

Exposure Group	Basal Respiration	Proton Leak	ATP-Linked ^b Respiration	Maximal Respiratory Capacity	Reserve Capacity ^c
Control	92.0 $\pm$ 6.0	47.0 $\pm$ 3.0	45	146.7 $\pm$ 7.2	55
AZT/3TC/NVP	75.0 $\pm$ 5.7	33.0 $\pm$ 2.7	42	153.7 $\pm$ 13.3	79
AZT/3TC/ABC	40.0 $\pm$ 5.7	19.0 $\pm$ 4.1	21	77.1 $\pm$ 3.8	27

^aValues, from 3–4 Seahorse plates/exposure group, represent 2 control patas, 3 patas exposed to AZT/3TC/NVP and 2 patas exposed to AZT/3TC/ABC. Patas given ARV drugs were exposed for the last 50% of gestation and for the first 6 weeks of life.

^bATP-Linked respiration is calculated by subtracting the proton leak (OCR with OLIGO) value from the basal value.

^cReserve capacity is calculated by subtracting the basal value from the maximal respiratory capacity value.